Biotechnology Effect of Protease Enzyme Produced From Lactobacillus Species on the Milk

Fekadu Mersha, Dr. Archana Pandita

Abstract— **In the world like developing countries there is rapid growth of human population and urbanization. This growing population uses numerous amounts of milk and milk products in day to day life. It demands to improve yield and quality of milk and milk products through biotechnological techniques like application of enzymes. This paper reviews the protease enzyme producing *Lactobacillus species*, production of protease enzyme and the effect of protease enzyme on milk and milk products. *Lactobacillus species* are the major part of the lactic acid bacterial groups. *Lactobacillus species* have function of pathogen control, probiotic, food producer (fermenter) and enzyme producer. Protease enzymes are hydrolyzed or breakdown peptide bonds of proteins and can break specific peptide bond or complete polypeptide chain to amino chain residues. They are used to apply in leather, pharmaceuticals, protein processing, foods, dairy, diagnostic reagents, soy processing, peptide synthesis and extraction of silver from used X-ray film. The production of protease is mainly affected by media composition, variation in C/N ratio, pH, temperature, and incubation time. Generally optimization of protease starts with conventional method. Conventional optimization method is simple and it supports to select main factors that accelerate production of protease. The major function to apply proteases in the dairy industry is associated with their ability to coagulate milk and to improve taste, flavor and texture in cheese manufacturing.**

Index Terms— **Lactic acid bacteria, Milk clotting, Protease enzyme, Probiotics, Optimization**

I. INTRODUCTION

Lactic acid bacteria have a number of genera with many species such as Lactobacillus, Pediococcus, Lactococcus, Leuconostoc etc(Dellaglio et. al., 2005). *Lactobacillus* is a genus of Gram-positive, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria. In humans, they constitute main microbiota at different body parts like digestive, urinary, and genital system (Pathak AP and Deshmukh KB. 2012). Presently, there are about 3000 different types of enzymes have been identified they give functions to apply in biotechnology and industries (Raposo, S., & Domingos, A. 2008). Proteases enzyme is hydrolyze peptide bonds of proteins and amino chain residues (Sharma, K. M. et. al., 2014), and this enzyme is classified into acidic, neutral and alkaline based on activity of its pH, alkaline proteases cover 89 % of the total protease sales (A.S. S. Ibrahim et. al., 2015). Proteases have a function in various industrial application like leather (Kanth, S.V. et. al., 2009) and food processing (Tavano, O.L., 2013). as well as in pharmaceutical (Brandelli, A. et. al., 2010). Conventional optimization is the simplest method that helps as a selection factors to enhance protease production (Neurath, H., 1984). Thus Increasing cheese production and consumption with the increment of calf rennet price in the world, led to replace calf rennet to proteases with coagulant properties in the manufacture of dairy products (Mazorra-Manzano, M. A. et. al., 2013). An application of proteases enzyme is important in the food and dairy industry with its ability to coagulate milk protein in cheese manufacturing (Mandujano-Gonzalez, V. et. al., 2013). The aim of this paper is to review the effect of protease enzyme that produced from *lactobacillus species* on the milk. That is used to improve the yield and quality of milk and milk product.

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Fekadu Mersha Department of Biotechnology, college of Enginerring and Technology, Sharda University

Dr. Archana Pandita, Assistant professor Department of Biotechnology, college of Enginerring and Technology, Sharda University

II. LACTOBACILLUS SPECIES

2.1 Property of Lactobacillus species

Lactobacillus species have mutual relation with the human body that protects the host against invasion of pathogens and the hosts provide nutrients (Martín R et. al., 2013). Currently there are about 180 species of Lactobacillus bacteria. http://www.bacterio.cict.fr/l/lactobacillus.html Some lactobacilli are present in the natural micro flora of the dairy products and originate from animals such as: *Lactobacillus casei. Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus curvatus, Lactobacillus acidophilus and Lactobacillus pentosus* (Gobbetti M. et. al., 2002). There are 10 species of lactobacillus bacteria those produce protease enzyme and they are listed under in the Table 1.

No	Name of bacterial species	References
1	*Lactobacillus delbrueckii*	(Shin JY. et.al., 2004).
2	*Lactobacillus casei*	(Shin JY. et.al., 2004).]
3	*Lactobacillus bulgaricus*	(Courtin P, et. al., 2002).
4	*Lactobacillus rhamnosus*	(Pastar I et. al., 2003).
5	*Lactobacillus paracasei*	(Bintsis T et. al., 2003).
6	*Lactobacillus helveticus*,	(Germond JE et. al., 2003).
7	*Lactobacillus brevis/ cellobiosus / fermentum / plantarum*	(Mugula JK. Et. al.,2003)

Table I. List of *Lactobacillus species* producing protease enzyme

2.2 Function of Lactobacillus

*Lactobacillusspecies*produce hydrogen peroxide organic acids and different metabolites these are controls the growth and virulence of the fungal pathogen *Candida albicans in vitro* and *in vivo*. (Vilela SF et. al., 2015). Both the presence of metabolites, such as sodium butyrate, and the decrease in environmental pH caused by the organic acids minimize the growth of hypha in *C.albicans*, which controll its pathogenicity (Vilela SF et. al., 2015). *Lactobacillus species* also minimizes the pathogenicity of C.albicans by controlling *C. albicans* biofilm formation (Vilela SF et. al., 2015). Administration of *Lactobacillus species* with other probiotics benefits cases of irritable bowel syndrome (IBS) and probiotics helps to treat IBS by returning homeostasis .(Ford AC, et. al., 2014) In addition, administration of *Lactobacillus species* as probiotics helps to control infections that occur by the ulcer-causing bacterium (Ruggiero, P. 2014). The probiotic micro-organisms which has been ingested in certain numbers exert health benefits beyond inherent nutrition has been added. (Klaenhammer, e. A. et. al., 2005), *Lactobacillus species* give support as starter culture in feed production to produce milk products and other food items (Inglin RC et. al., 2015). Production of bacteriocins to inhibit microorganisms is used as a base for antibacterial and antifungal activity of *Lactobacillus species* (Cribby S, et. al., 2009).

III. Protease Enzyme

3.1 Sources of protease enzyme

There are different sources of protease enzymes like microbial (Yegin, S., et. al., 2012). animal (D'ambrosio, A. et. al., 2003). and plant (Raposo, S., & Domingos, A. 2008). Microbial proteases have afunction to catalyse the hydrolysis of proteins to peptides and amino acids. They contributes about 40% production and 60% sales of enzyme for the total world (Novelli, P.K., et. al., 2016). Fungi have a contribution for various industries due to their ability to produce various extracellular enzymes. They are more advantageous than bacteria, because of their simpler downstream processing, lower material costs, higher productivity, faster production, and easier modification of enzymes (Hajji, M. et. al., 2010). Carbon and nitrogen sources, temperature and pH, agitation and incubation time are influences extracellular production and activity of fungi proteases (Belmessikh, A. et. al., 2013). Filamentous fungi production of acid protease and their demand is growing quickly (Aleksieva, P.and Peeva, L., 2000). *Lactobacillus species* are very important bacteria which have been belonging to the producers of protease enzymes for industrial application and different research purposes (Widsten, P., & Kandelbauer, A. 2008). The pervious researches and experimental studies views on Bacillus *species* had shown the maximum protease production. For e.g. two Lactobacillus strains such as *Lactobacillus homohiochii* and *Lactobacillus curvatus* were isolated from a Portuguese traditional dry fermented sausage have been seen high protease production and they have tyrene and ornithinede carboxylase activities (Olajuyigbe, F.M., and Ajele, J.O., 2005). It have been studied that alkaline protease from *Bacillus subtilis* and showed that pH 10 and temperature of 45 ºC were optimum conditions for the best activity of protease enzyme (Pant G, et al., 2015). But in pseudomonas alkaline protease has been active at pH 9 and temperature of 45 ºC [70]. (Meena P, et. al., 2013). Pepsin, chymotr ypsin, rennin, and pancreatic trypsin are proteases from animal sources. Trypsin is located in the intestine used as digestive enzyme of human being that hydrolysis food proteins, however chymotrypsin is found in the pancreatic extracts of animals and pure chymotrypsin is so expensive, it is used in diagnostic and analytical applications (Rao, M. B. et. al., 1998).. Pepsin is found in the stomach of vertebrates it is an acidic protease. Pepsin is now being replaced by a mixture of serine and metal microbial proteases (Adinarayana and Ellaiah, P. 2002). Natural rennet is one of animal protease that obtained from abomasums of calves and is mainly produced by chymosin and pepsin (Mazorra-Manzano, M. A. et. al., 2013). Due to their compatibility for biotechnological applications plant proteases have different benefits (H.Uhlig 1998). However, it is insignificant in terms of quantity that the commercial application of plant proteases (Aehle, W., 2004). The investigation has been done to search out the novel protease enzyme from the leaves of medicinal plant that have a function of therapeutical agent and industrial applications (Chinnadurai, G. S., et. al., 2018). Plant proteases exhibited more pH 7 and are active at neutral pH. They do not require cofactors and preferred for industrial application (Godlewski, M.,and Adamczyk, B., 2007).

3.2 Classification of protease

Proteases enzyme has different classification such as serine, therionin, cysteine, glutamic and aspartic proteases depending upon the amino acids present in the active site, or as metalloproteases if a metal ion is needed for their catalytic activity (Rawlings, N.D.et. al., 2014). Protease enzymes produced from *Bacillus sphaericus* NRC 24 (El-Bendary, M. A. et. al., 2007). and *Bacillus licheniformis* USC13 (Ageitos, J. M. et. al., 2007). have been identified as serine proteases (SPs). Serine proteases are so named due to the fact that they have catalytic serine residue at the active site, the catalytic mechanism provides more residues that are proton donors comprising approximately 2.5% of the whole genome (Young, N.D.et. al., 2012) In the genomes of S. mansoni or C. sinensis, almost 25% or 30% of all proteases, respectively, classified as SPs (Howe, K.L. et. al., 2017). Threonine protease is another industrially significant enzyme where its residue lies on catalytic site. The main two threonine protease superfamilies grouped in to Ntn fold proteosomes and DOM fold ornithine acyltransferases (Mala, B. R. et. al., 1998).

Cysteine proteases (Cp) have a function of development and ripening fruits, nutritional reserve, degradation of storage protein in germinating seeds, activation of proenzymes and degradation of defective proteins. They are found in various organisms (Turk, V., et. al., 2012). The glutamic protease is classified to six catalytic type of peptidase at present it has peptidases from five species of Ascomycota. Recent analysis of the molecular structure and catalytic mechanism for this glutamic protease has identified these enzymes as a novel protease family, the Eqolisins, a name obtained from the active-site residues, glutamic acid (E) and glutamine (Q) (Fujinaga, M., et. al., 2004). Members of this newly identified family of peptidases have a previously undescribed ß-sandwich as a tertiary fold and a unique catalytic dyad consisting of glutamine and glutamate residues (Fujinaga, M., et. al., 2004). Glutamic proteases have been shown to be

responsible for degrading recombinant proteins (Moralejo, F.J. et. al., 2002).

Aspartic proteases are a subfamily of endopeptidases that have been isolated from several sources (Mandujano-Gonzalez, V.et. al., 2013). They are active in acidic conditions (pH 3-5) and inhibited by pepstatin A (Chen, J. et. al., 2009b). Aspartic proteases are used in cheese production due to their milk-clotting property. Chymosin is an aspartic protease and a common milk-clotting enzyme that mainly cleaves κ-casein and accelerates milk coagulation in cheese production (Majumder, R. et. al., 2015)Metalloprotease enzyme is more fruitfull in cleave gelatin and large globular protein azocasein. It has been further characterised by 2DE combined with 2DZ. The MCA/PA ratio obtained by the purified metalloprotease of T. clypeatus was similar to that obtained for the commercial preparation. Milk-clotting enzyme with its specificity to the b-casein was purified and identified as a metalloprotease from *Bacillus subtilis* and *Bacillus amyloliquefaciens* D4 (Li, Y. et. al., 2012).

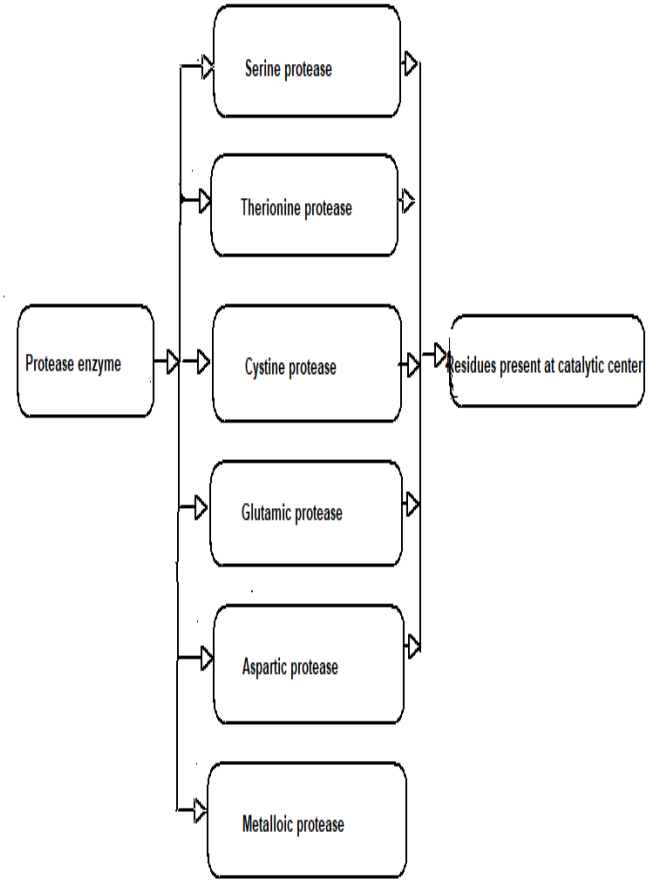

Fig 1 Diagrammatic representation of protease Classification (https://www.google.co.in/search?biw=1366&bih)

3.3 Functions of protease enzyme

Protease enzymes have various industrial functions to apply in leather, pharmaceuticals, protein processing, food processing, dairy processing, diagnostic reagents, soy processing, peptide synthesis industries, and extraction of silver from used X-ray film (Kumar RS et. al., 2014). Alkaline proteases are used for several bioengineering and biotechnological applications and account for approximately 30% of the total world enzyme production (Haddar A. et. al., 2009). It has a function in detergent formulations as cleaning additives to accelerate the breakdown and release of proteins (Joshi S. and Satyanarayana T. 2013). The powder form of protease in detergent being prepared as wax–containing granules that safe user from undesirable inhalation of protease dusts (Ward, O. P. 2011). The use of crude protease from *Bacillus licheniformis* LBA 46 on washing performance of cotton fabrics, and detergents have been heated for 30 min at 95 ºC to discared enzymatic activity presented (Contesini, F. J. 2014). Proteases in leather processing have a function of substitutes to traditional processing used toxic and dangerous chemical products (S. Sivasubramanian et. al., 2008). Collagen is main leather protein that's why some leather processing requires removal of non-collagenous constituents. The degree to apply these modifications controls the physical characteristics of the leather (E.H.A. Nashy et. al., 2005) Proteases are important to improve dehairing, degrade non-collagenous constituents of the skin and eliminate non-fibrillar proteins (Jisha, V. N. et. al., 2013). Alkaline proteases are most stable and active to remove hair and they reduce the immersion time by facilitating a faster absorption of water (Jisha, V. N. et. al., 2013).The optimization of protease for unhairing leather process from *Bacillus subtilis* was identified previously by various research works(Dettmer, A. et. al., 2012). Proteases function has been studied in degrading gelatin coating over the used X-ray and photographic films that separates silver present in the gelatin layer. About 18–20% of the world's silver derived from waste products like X-ray and photographic films (Nakiboglu, N. et. al., 2003). Proteases in the food industry have many functions such as production of dairy products, bakery and clarification of xanthan gum and others. Bioactive hydrolysates produced during proteolysis of ovine casein and protease is important to improve dairy products through milk-clotting processes (Daroit, D. J. et. al.,2012). In cheese production proteases are used as milk-clotting agents and to improve cheese quality like taste, flavor, texture and functional properties (Yegin, S. et. al., 2011). Proteolysis has a responsibility to biochemical modifications of cheese making A purified protease from *Pseudomonas fluorescens* RO98 hydrolyse bitter peptides in Cheddar and Gouda cheese in a reaction of 90 min at 30 ºC and pH 6.8 . (Koka, R. & Weimer, B. C. 2000). Protease is also very important enzyme to apply in the textile industry and the protease removal of sericin is an environment-friendly process of silk degumming (Arami, M., et. al., 2007)]. Applying proteases to wool shrink-resistance process improve whiteness, dye-ability and handling. (Ibrahim, N.A. et. al., 2012). Acommercial protease covalently linked to Eudragit S-100 for wool shrink-resist finishing, replacing the conventional chlorine treatments (Shen, J., et. al., 2007). However, enzymatic treatment may cause damages to the fiber cuticle and losses weight and strength of wool fiber (Ibrahim, N.A. et. al., 2012). The immobilization of proteases typically increases their molecular size, helps to produce a higher tensile strength and an optimum felting of the fibers (Araujo, R. et. al., 2009).

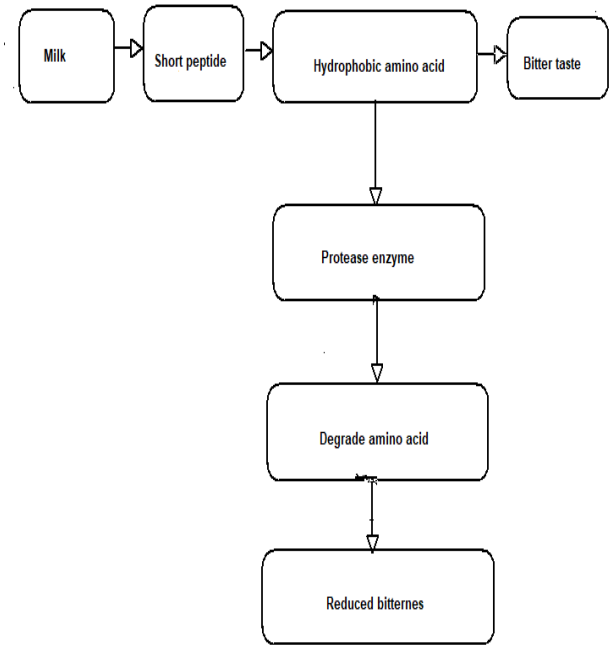

Fig 2 Diagramatoic representation of protease function to improve milk taste
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IV. PROTEASE PRODUCTION

The effect of protease treatment on wettability and dyeability of nylon blended fabric was also studied recently (Waly, A.I. et. al., 2016). Acid proteases have been investigated in skin associated with desquamation and the ability to induce exfoliation of potential active compounds in cosmetic products (Smith, W.P. et. al., 2007). Proteases have also been tested as biocontrol agents against numerous agricultural pests and root knot nematodes (Ward, E., et. al., Kerry, B.R., Manzanilla-Lopez, R.H., Mutua, G., Devonshire, J., Kimenju, J., Hirsch, P.R., (2012).

The protease enzyme produced by Sporisorium reilianum has been obtained maximum yield at 96 h during the stationary phase of growth in acidic conditions [67]. (Mandujano-Gonzalez, V. et. al., 2013). The protease produced from A. niger and Aspergillus fumigatus has been exhibited maximum yield in the stationary phase after 144 h and 48 h of fermentation, respectively [34]. (Farnell, E.et. al., 2012). The protease enzyme to be used as a catalytic agent in organic solvents was studied by different research scholars [35]. (Fang, Y. et. al., 2009). The use of casein as the substrate under the standard assay conditions gave the highest activity at pH 8.0 [26]. (D. Karadag, et. al., 2009). Other experiments have been showed that an incu-bation temperature of 37 ◦C, pH 9.0, carbon and nitrogen source medium are resulted in the maximum production of protease [1]. .(S. Qureshi et. al., 2011). The production of protease enzyme yields vary considerably based on the temperature and pH [99]. (V. Maghsoodi, A.et. al., 2013.) In the production process of protease enzyme that the ability to utilize a given carbon or nitrogen source is differs from one microorganism to another one[108]. (Hajji, M. et. al., 2008).

Table II. Characteristics of protease enzyme in production process. [96]. (Sumantha, A. et. al., 2006b).

No	Parameters	Serine proteases	Metallo proteases	Aspartic proteases	Cysteine proteases
1	Molar mass (kDa)	18 – 35	19 – 37	30 – 45	34 – 35
2	Optimum range of pH	6 – 11	5 – 7	3 – 5	2 – 3
3	Optimum range of Te. (ºC)	50 – 70	65 – 85	40 – 55	40 – 55

4.1. Protease optimization

It is essential to optimize production medium and cultivation conditions for the protease growth and enzyme production, it helps to obtain maximum and commercial yield of protease (Deng, A. et. al., 2010). There are different parameters used for protease optimization such as Carbon, Nitrogen, metal ion & salt, pH and Temperature. The alkaline protease production has been depends on concentrations of fructose as carbon source. Both growth and alkaline protease production have been increased by increasing the fructose concentration. Although further increases in fructose concentration led to the decrease the yield of enzyme production (Pathak AP and Deshmukh KB. 2012). The use of maximum yeast extract incurs higher protease enzyme production. However, the protease production my decrease by using more extra yeast extracts and other inorganic nitrogen sources (Chu WH. 2007). It has been found that other organic nitrogen sources supporte d protease production including skim milk (Gouda, M. K. 2006). peptone (Oskouie SFG et. al., 2008). casamino acids (Jain D et. al., 2012). beef extract (Kumar RS et. al., 2014)., and others (Jain D et. al., 2012). The previous studies has been shown that an increasing salt concentration causes change in the lipid resulting a decrease of growth rate that make reduced enzyme production (Chandran S et. al., 2005). Increasing the salt concentration to 7.5% causes a relative decrease in both bacterial growth and protease production to 78% and 85.9% of the higher enzyme yield and cell growth (Jain D et.al., 2012). Calcium and Ba metal ion has been reported to enhance protease production in several organisms (Nadeem M et. al., 2007). It has been reported that in various studies these metal ions protect enzyme from thermal denaturation and maintain its high temperature (Joshi RH et. al., 2008). At pH 5–7, the bacterial growth and protease production were significantly reduced. (Horikoshi K. et. al., 2011). The optimum pH is 9 - 10 for growth and protease production and it is common for alkaliphilic and haloalkaliphilic organisms. (Pathak AP and Deshmukh KB. 2012). Protease productions by strain NPST-AK15 has been studied at different growth temperatures (30–60°C). The optimum temperature for protease production was found to be at 40°C. There has been a drastic decrease in the enzyme

production at higher temperatures that the enzyme yield reduced to 26.2% while 10.1% of the maximum production at 45°C and 50°C, respectively (Ibrahim, A. S.et.al., 2015)..

4.2 Purification of protease

Protease enzyme purification is used for the product recovery and there are different techniques to purify protease enzyme. An experiment has been indicated that polyacrylamide gel electrophoresis was a method used to purify the protease produced by Conidio boluscoronatus (Neurath, H., 1984). The purification of thermostabl serine alkaline protease enzyme from B. subtilis PE 11through filtration chromatography with the sephadex G 200 column method was studied different research institutions (Adinarayana,K. et.al., 2003). A solvent stable alkalophilic protease enzyme purification from B. subtilis TKU007 by DEAE-Sephar- ose CL63 and Sephary lS 100 chromatography was investigated by many researchers (Lang Wang, S.,and XiYeh, P., 2006). The purification and characterization of the alkaline protease from Bacillus species, by using Phenyl-sepharose column (Chang,C.S. et. al., 2004). The proteases enzyme produced from selected bacterial strains have been purified by two different methods viz. sodium alginate MLP-TPP method and ammonium sulfate–Butanol precipitation (ASBP) method (Prakash, S. et. al., 2014). Purification and characterization of keratinolytic protease enzyme has a function of subtilisin-like serine protease that having a mass of 26 kDa that hydrolyses casein at high rates (Daroit, D. J. et. al., 2012). There has been qualitative and quantitative variation of purified protease enzymes inall the above different purification methods.

V. THE EFFECT OF PROTEASE ENZYME ON MILK CLOTTING

The protease enzymes are capable of clotting milk while chymosin-like proteases are more suitable due to their specificity and purity (Merheb-Dini, C. et. al., 2010). Aspartic proteases are playing agreate role as milk coagulating enzymes in cheese production. Milk coagulation is a process that related to the hydrolysis of κ-casein by enzymes destabilizing casein micelles ((N.-W. Hsiao et. al., 2014). Protease enzyme has major proteolytic activity that shortens the age course of cheese [10]. (An, Z. et. al., 2014). and it improves the flavor, taste and texture of cheese (Abbas, H. et. al., 2013). The degree of CN hydrolysis positively influence the cheese meltability and negatively influence hardness of the cheese, However the extent of CN hydrolysis have positive impact to cheese hardness and negative impact to cheese meltability . (Kim, S. Y. et. al., 2004). It has been showed that the specific cleavage site for bovine-CN is the Phe105–Met106 bond for chymosin and aspartic acid proteases . (Jacob, M.et. al., 2011). Those different cleavage sites do not seem to affect clotting the milk . (Egito, A. S. et. al., 2007). The application of MCEs from T. indicae-seudaticae N31 . (Alves, L. S. et. al.,2013). and C. parasitica . (Kim, S. Y. et. al., 2004). Many research results shows that enzyme concentration affects MCA and that milk-clotting time is minimized as the enzyme concentration increased . (Beka, R. G. et. al., 2014). The sensory attributes and textural pramater of milk products is mostly depends on the optimal coagulant property of protease enzymes (Børsting, M. W.et. al., 2015).

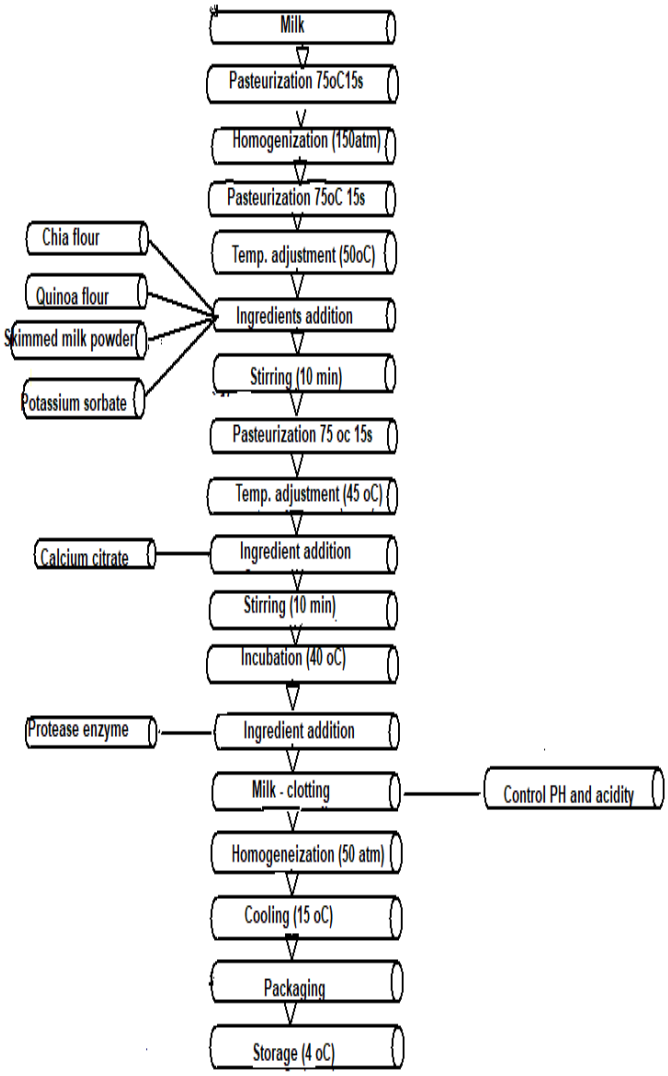

Fig 3 Diagramatic representation of cheese making using protease [63].(Lemes, A. C.,et. al., 2016).

CONCLUSION

This review gives an idea for the proteases and mainly focused on the general aspects of proteases giving special emphasis from *Bacillus species. Lactobacillus species* used as pathogen control, probiotics, food and enzyme producer. Also protease has different industrial functions like detergent formulations as cleaning additives, important to improve dehairing of leather, degrading gelatin coating over the used X-ray to separates silver from gelatin layer, production of dairy products, bakery and clarification of xanthan gum, wool shrink-resistance process improve whiteness in textile, induce exfoliation of potential active compounds in cosmetic products and as biocontrol agents against numerous agricultural pests and root knot nematodes. It needs to understand the production of protease enzyme from *lactobacillus species* and also the function of protease on different industrial application and its effect on milk and milk products. Many experiments showed that an incubation temperature of 37 ◦C, pH 9.0 and glucose and sodium nitrate as the carbon and nitrogen source, respectively, resulted in the

highest production of protease. It is essential to optimize production medium and cultivation conditions for the growth and enzyme production. Protease is the major milk clotting enzyme and it is utilized for accelerating cheese rippenening, good flovour and textural development. The desirable attributes of this protease enzyme is to improve yield and quality of milk and milk product, and it could be achieved by partial and gradual break down of carbohydrates, lipids, and protein during clotting and ripening of milk and milk products.\

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